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GERMPLASM AND MOLECULAR MARKERS FOR DISEASE RESISTANCE IN POTATO

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This application claims priority to U.S. Provisional Application Serial No. 60/054,267, filed July 30, 1997, which is incorporated by reference herein in its entirety.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the United States Department of Agriculture.

FIELD OF THE INVENTION

This invention relates to the field of genetic manipulation of higher plants. More specifically, the invention relates to novel germplasms, breeding stocks and molecular markers created or identified by somatic fusion of domestic and wild potato species, which are useful for development of potato varieties resistant to late blight and other fungal pathogens.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application in parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

Plant diseases cause billions of dollars in losses to farmers in the United States and elsewhere in the world every year. Generating crop plants that are naturally resistant to disease has been a goal of plant breeders for decades. Classical breeding methods have been supplemented in recent years by molecular genetic

techniques, e.g., to identify a gene that encodes a protein with antifungal or antibacterial properties (often not a plant gene) and then express this gene at high levels in a plant.

Another approach is to use genes from wild species to improve disease resistance and other agronomic characteristics of cultivated crops. For the most part, however, breeders have been restricted to those genetic combinations that can be obtained by direct sexual crosses or through bridging crosses through several species.

Protoplast fusion in some cases has provided a wider range of available genes. By this technique, the somatic cells of two species are combined. Plants can then be regenerated from the combinations and examined for the expression of the desired attributes and for fertility. In this manner it is possible to incorporate useful traits from widely separated, sexually incompatible, species into breeding lines. Using other molecular techniques, it is also possible to identify genes in those wild species responsible for conferring the useful trait, such as resistance to one or more plant pathogens. The genes can then be incorporated into the genomes of a variety of different species to develop resistance to one or more plant pathogens.

Potato (*Solanum tuberosum*) is the world's fourth most valuable crop. In the United States, the value of the crop exceeds two billion dollars each year. In spite of its high value, the commercial crops are subject to many disease problems, including foliar diseases such as late blight and early blight, virus diseases, soil problems such as those caused by nematodes or *Verticillium* species, and bacterial diseases such as bacterial wilt (in the field) or *Erwinia* soft rot (in storage). These diseases are costly in terms of crop loss, expenses associated with application of chemicals and environmental impact of pesticide use. Such costs

could be minimized or avoided if resistant potato varieties were available. However, adequate resistance for late blight, *Erwinia* soft rot and many other diseases has not been incorporated into potato cultivars, partly because of the lack of a good diversity of resistance genes that breeders can use to develop resistant cultivars.

Of particular importance is the late blight disease, cause by the fungus *Phytophthora infestans*. Late blight remains one of the most devastating diseases of potatoes worldwide. Despite its importance, no major cultivars with adequate late blight resistance are grown in the United states today. Until recently, crops were protected from the disease by cultural methods (e.g. crop rotation, crop hygiene) and with fungicides. The absence of compatible mating types within the U.S. heretofore has prevented sexual recombination. However, a second mating type has now become widespread in the U.S. and many lines of the fungus have become resistant to one of the key, very effective, systemic fungicides (Metalaxyl) registered for potato late blight control.

The late blight fungus is also a devastating pathogen on crops other than potato. It infects tomatoes, eggplants and other solanaceous species. Other *Phytophthora* species are pathogenic to a wide array of agronomically important plants, including grapes, avocados and several varieties of fruit and nut trees. Accordingly, a source of resistance to *Phytophthora* species that could be introduced into these species by molecular genetic techniques would also be of great value.

Possible sources of resistance to many potato pathogens exist in related *Solanum* species. Several *Solanum* species have been crossed with the cultivated potato in an effort to introgress disease-resistance genes.

When sexual crossing techniques have failed,

disease resistance transfers have been attempted with somatic cell fusions. Potato protoplasts have been fused with a number of sexually incompatible wild *Solanum* species (e.g., *S. brevidens, S. bulbocastanum, S. commersonii, S. polyadenium, S. etuburosum)*, and many fertile somatic hybrids have been regenerated (Austin et. al., 1985, 1993; Ehlenfeldt & Helgeson, 1987; Kim-Lee et al., 1993; Novy & Helgeson, 1994b). Somatic hybrids have been screened for useful disease resistance (Helgeson et al., 1986; Austin et al., 1988; Novy & Helgeson, 1994b) and these resistances are heritable (Helgeson et al., 1993).

Solanum bulbocastanum is a particularly desirable wild species from which to seek useful disease resistance genetic traits, inasmuch as it exhibits resistance to several potato pathogens, including nematodes, early blight, late blight and *Verticillium*. Disease- or pest-resistant somatic hybrids of *S. bulbocastanum* and cultivated potato have been produced by the present inventors and by others. In one instance, analysis of BC1 and BC2 progeny of a nematode-resistant *S. bulbocastanum* - *S. tuberosum* somatic hybrid revealed that the nematode resistance locus is likely to reside on chromosome 11 of *S. bulbocastanum* (Brown et al., 1996). However, the chromosomal location of late blight resistance in *S. bulbocastanum* heretofore has not been identified.

Clearly, there is an ongoing need to identify such resistances in wild potato species, such as *S. bulbocastanum*, to continue improving the disease resistant characteristics of cultivated potatoes. A need also exists to identify the genes in wild potato species that are responsible for disease resistance. Once isolated, these genes can then be introduced by molecular genetic techniques into species other than potato to confer resistance to one or more plant pathogens.

SUMMARY OF THE INVENTION

This invention provides novel germplasms, breeding stocks, molecular markers and methods for introducing late blight resistance into cultivated potato plants. The invention further provides genomic DNA segments from *S. bulbocastanum* useful for introducing resistance to the late blight fungus, *Phytophthora infestans*, into species other than potato.

According to one aspect of the invention, a potato germplasm is provided that confers resistance to the late blight fungus, *Phytophthora infestans*, as well as other fungal pathogens, including early blight, *Erwinia* soft rot and *Verticillium*. The most fundamental form of this germplasm is a tissue culture produced by somatic hybridization of *S. tuberosum* with *S. bulbocastanum*. Fertile plants regenerated from these hybrids are also provided, along with progeny resulting from crosses with agronomically preferable cultivated potato species.

According to another aspect of the invention, a late blight-resistant potato plant is provided, comprising a segment of a genome from *Solanum bulbocastanum* which contains a gene that confers resistance to late blight. In a preferred embodiment, the genomic segment of *S. bulbocastanum* is from chromosome 8, and co-segregates with one or more of the following markers: (1) a RAPD marker referred to herein as $GO2_{586}$; (2) a RAPD marker referred to herein as $PO9_{587}$; and (3) RFLP marker CT88, RFLP marker, RFLP marker CT148, RFLP marker CT252 and RFLP marker CT68. The potato plant may also be resistant to at least one additional disease, such as potato early blight, *Erwinia* soft rot, and *Verticillium* wilt.

In a preferred embodiment, the aforementioned late blight resistance gene is incorporated into the potato plant by somatic hybridization between a cell of a parent of the plant and a cell of *Solanum bulbocastanum*.

In another embodiment, the late blight resistance gene is incorporated into the plant by genetic transformation of a cell of the plant with a plant transformation vector containing the gene.

According to another aspect of the invention, an isolated nucleic acid molecule is provided, which is complementary to part or all of a 0.6 kb segment of a *Solanum bulbocastanum* genome, which co-segregates with a gene that confers resistance to late blight. In a preferred embodiment, the segment comprises part or all of the RAPD marker $G02_{586}$ or $P09_{587}$, having the sequence of SEQ ID NO:1 or SEQ ID NO:2, respectively.

According to another aspect of the invention, a method of monitoring late blight resistance in a breeding cross of progeny of a fertile somatic hybrid of *Solanum tuberosum* and *Solanum bulbocastanum* is provided. The method comprises: (a) performing the cross; (b) isolating genomic DNA from individual progeny of the cross; and (c) detecting in the genomic DNA the presence or absence of a genetic marker that is pre-determined to co-segregate with the late blight resistance, the presence or absence of the marker being indicative of the presence or absence of the late blight resistance in the individual progeny of the breeding cross. In a preferred embodiment, the marker is selected from the group of RAPD and RFLP markers listed above.

According to yet another aspect of the invention, a method of identifying a *Solanum bulbocastanum* gene that confers resistance to late blight is provided. The method comprises: (a) cloning a DNA segment that co-segregates with the late blight resistance phenotype in progeny of somatic hybrids of *Solanum bulbocastanum* and *Solanum tuberosum*; (b) providing a genomic library of the *Solanum bulbocastanum* genome; (c) isolating clones of the genomic library that contain segments which hybridize with the co-segregating DNA segment; and (d) identifying at least one gene

disposed within the isolated genomic clones that confers the late blight resistance. In a preferred embodiment, the cloned DNA segment that co-segregates with late blight resistance comprises part or all of one of the RAPD or RFLP markers listed above.

According to further aspects of the invention, a late blight resistance gene from *Solanum bulbocastanum*, produced by the aforementioned method, is provided. Also provided is a transgenic plant comprising the resistance gene.

The advantages of the present invention with be better understood in view of the detailed description and examples set forth below.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1. RFLP analysis of somatic hybrids between *S. bulbocastanum* and potato. The probe used in the analysis was TG310, a tomato genomic probe specific for Chromosome 1 of tomato and potato.

Figure 2. MapMaker analysis of chromosome 8 of *Solanum bulbocastanum* with RAPD (randomly amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) markers and resistance to late blight. Percentages in parentheses (left column) indicate recombination frequencies calculated by dividing the deviation from complete co-inheritance by the population size. Numbers to the right of the percentage column indicate distances between markers in centimorgans. Numbers in parentheses to the right of the chromosome 8 diagram represent the arbitrary code number of the individual in the population. The far right column lists RAPD markers, which are named by the decameric primer and the size of the amplified transcript (e.g. "GO2-586" or “PO9-587") and RFLP markers. The resistance locus is indicated by “R”. Map scale is 10.0 cM per 1.21 cm.

Figure 3. Tomato chromosome 8 map, after Tanksley et al., 1992, showing approximate corresponding

location of *S. bulbocastanum* late blight resistance gene(s) on tomato chromosome 8.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, genetic markers have been identified that are linked to late blight resistance derived from somatic hybrids of *Solanum bulbocastanum* and cultivated potato (*Solanum tuberosum*). Backcross (BC_2) populations were analyzed for resistance to late blight and for randomly amplified polymorphic DNA (RAPD) and RFLP markers. Three test populations were derived from two different somatic hybrids between potato and *S. bulbocastanum*. Each somatic hybrid was crossed with the cultivar Katahdin to generate BC_1 parents, all of which were resistant to late blight. BC_1 progeny were crossed with three different potato breeding lines (Norland, Atlantic and A 89804-7), all of which are sensitive to the late blight fungus. Each BC_2 population contained more than 50 individuals and segregated for resistance to late blight. In each population, late blight resistance was correlated (>95%) with the presence of a RAPD marker ("$GO2_{586}$") keyed to chromosome 8 of *S. bulbocastanum*. The identification of this novel molecular marker is described in greater detail in Example 2.

Further genetic analysis has led to the identification of another RAPD marker and several RFLP markers that are also associated with the resistance gene or genes of *S. bulbocastanum* chromosome 8. These include, for example, RFLP marker CT88 and RAPD marker $PO9_{587}$, which appear to flank the resistance locus (see Figure 2). Other closely linked RFLPs include CT148, CT252 and CT68 (Figure 2). Together, these markers define a specific location on *S. bulbocastanum* chromosome 8 that carries the gene(s) which confer resistance to late blight and certain other potato diseases, as discussed in greater detail herein. The approximate

location of the resistance genes on Chromosome 8 (as mapped by Tanksley et al., 1992) is shown in Figure 3.

This invention provides a new and useful germplasm and breeding stock for breeding potato cultivars with resistance to late blight. The germplasm comprises fertile hybrids produced by somatic fusion of *S. bulbocastanum* with *S. tuberosum*, and progeny thereof, which contain that portion of the *S. bulbocastanum* genome carrying the late blight resistance gene or genes. The presence of this genomic fragment is conveniently monitored by the presence of closely linked RAPD markers, $GO2_{586}$ or $PO9_{587}$, or closely-linked RFLP markers, such as CT88, as described in greater detail below. Particularly preferred breeding stock is obtained by repeated backcrosses of the somatic hybrid with potato cultivars having desirable agronomic qualities, with the presence of the late blight resistance-conferring genomic segment of *S. bulbocastanum* being monitored by detection of one or more of the relevant RAPD or RFLP markers.

Creation of *S. tuberosum* - *S. bulbocastanum* somatic hybrids, selection of fertile, resistant plants, and production of subsequent backcross generations comprising the disease resistance gene or genes are all accomplished by methods well known to plant breeders and molecular biologists. Preferred methods are described in greater detail in Example 1.

This invention also provides novel molecular markers to facilitate selection of breeding progeny that contain the resistance-conferring genomic segment from *S. bulbocastanum,* without having to perform field or greenhouse trials for disease resistance. One closely linked marker, $GO2_{586}$, was created through the use of RAPD markers, using commercially available oligonucleotide 10-mers as primers for PCR amplification. RAPD markers are generated by incubating genomic DNA with a population of 10-mers under conditions that allow the oligonucleotides to bind to any complementary sequences in the genomic

DNA. The length of DNA between two bound sets of primers is amplified by PCR, thereby generating a DNA segment which is a copy of the segment between the primers in the genomic DNA, i.e. a RAPD fragment. The nucleotide sequence of the $GO2_{586}$ RAPD marker is set forth herein as SEQ ID NO:1.

RAPD fragments thus created are species specific markers which can be keyed to particular chromosomes by comparative RFLP analysis and can be followed as dominant markers through various crosses. In accordance with the present invention, RAPD marker $GO2_{586}$ (which is a 586 bp fragment primed by the "GO2" decameric oligonucleotide purchased from Operon Technologies, Inc.) was keyed to *S. bulbocastanum* chromosome 8. Analysis of BC_2 progeny of potato-*S. bulbocastanum* somatic hybrids demonstrated that late blight resistance correlates with greater than 95% frequency with the presence of the $GO2_{586}$ RAPD fragment. This tight segregation of late blight resistance with the marker indicates that the resistance gene or genes reside at or near the location of the marker. Accordingly, late blight resistance in further crosses can be monitored by detecting the presence or absence of the marker. In this manner, progeny having a high probability of carrying the resistance gene(s) can be selected without lengthy greenhouse or field trials for disease resistance, thus making the breeding process faster and more efficient.

This invention also provides a second RAPD marker, $PO9_{587}$, which is also closely linked with the resistance gene(s) in *S. bulbocastanum*, and which was identified by the same protocol as set forth above for $GO2_{586}$. The nucleotide sequence of RAPD marker $PO9_{587}$ is set forth herein as SEQ ID NO:2.

This invention further provides RFLP molecular markers, useful in facilitating selection of breeding progeny that contain the resistance-conferring segment of *S. bulbocastanum*. These markers can also assist in

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defining the location of the resistance genes on the chromosome and obtaining isolated genomic segments containing the gene(s). The nucleotide sequence of RFLP CT88 from three different sources (published by Tanksley et al. (http://probe.nalusda.gov:8300/cgi-bin/browse/solgenes), from R4 potato, and from *S. bulbocastanum*) are set forth herein as SEQ ID NOS: 3, 4 and 5 respectively).

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Once created, useful RAPD or RFLP fragments can be maintained in any suitable cloning vector. For instance, the $GO2_{586}$ marker generated in accordance with the present invention is maintained in a plasmid vector provided with a commercially available PCR kit (Invitrogen, Inc.). It is noted that obtaining RAPD should be replicable by anyone of skill in the art, using the commercially available decamers and the methods described in Example 2 and references cited therein. Alternatively, RAPD fragments described herein can be duplicated by nucleotide synthesis using standard methodologies.

The RAPD fragments, or portions thereof, or any of the linked RFLPs discussed above or shown in Figure 2, are used to monitor the presence or absence of the late blight resistance gene(s) by labeling them as probes to hybridize with complementary sequences in the *S. bulbocastanum* genome. The cloned fragments may be labeled according to any standard means, many of which are set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1997. The complementary genomic DNA is detected by one of several standard methods including, but not limited to,(1) *in situ* hybridization; (2) Southern hybridization (3) "dot blot" hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR). Detection of the complementary genomic DNA is indicative of the presence of the late blight resistance-conferring gene(s), due to the close linkage

of the R gene(s) and the RAPD marker or RFLPs, as discussed above.

The RAPD fragments, or portions thereof, or any of the aforementioned RFLP fragments, may also be used to identify and isolate the closely-linked late blight resistance gene of *S. bulbocastanum*, using methods well known to molecular biologists. In a preferred embodiment, a genomic library of *S. bulbocastanum* is constructed a suitable cloning vehicle, e.g. cosmid, yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC). The library is then screened by hybridization with the cloned RAPD fragment and/or one or more of the RFLP markers, and hybridizing clones are isolated. The hybridizing clones are then further analyzed, e.g. by mapping, sequencing and transcript analysis, to identify candidate open reading frames that may encode the resistance factor(s). Once candidate open reading frames are identified, they in turn may be further analyzed (e.g. by construction and *in vitro* expression of a cDNA molecule) in order to characterize the resistance gene and its encoded protein.

The disease resistance-conferring clone thus identified may be used to introduce late blight resistance into cultivated potato by molecular genetic techniques. For example, a binary bacterial artificial chromosome (BIBAC) vector may be used to mobilize a BAC genomic insert into potato (the vector BIBAC2 (Hamilton et al, 1996) has been used to mobilize large (>150 kb) DNA inserts into tobacco). A BIBAC vector containing the relevant genomic insert from *S. bulbocastanum* can be transferred into an *Agrobacterium tumefaciens* and used to transform selected potato cultivars. Alternatively, potato cells can be transformed via biolistic delivery of the BIBAC clone. Putative transgenic clones can then be evaluated by standard methods to determine if the transformation has been successful.

The disease resistance-conferring clone also

may be used to introduce resistance to *Phytophthora infestans* in species other than potato, which are sensitive to infection by that organism. These species include, but are not limited to, tomato, eggplant and other *Solanum* species. Furthermore, it is possible that the late blight resistance gene(s) of *S. bulbocastanum* may confer resistance to diseases other than late blight, and so may be of even broader utility for introducing disease resistance in potato and other plant species. The gene may be particularly useful for introducing resistance to other *Phytophthora* species pathogenic to grapes, avocados, fruit trees and nut trees. Moreover, once the gene's function has been determined, this may lead to the discernment of new mechanisms of resistance in other species.

The following examples are provided to describe the invention in greater detail. They are intended to exemplify, not to limit, the scope of this invention.

EXAMPLE 1
Resistance to Late Blight in Somatic Hybrids of *Solanum bulbocastanum* and Potato, and Progeny Thereof

A Mexican wild species, *S. bulbocastanum*, is highly resistant to late blight. However, *S. bulbocastanum* is a 1EBN species and thus extremely difficult to cross directly with potato.

Somatic hybridization can provide a means of bypassing sexual incompatibility between Solanum species, leading to fertile plants that can be used directly in breeding programs. The experimental results set forth in this Example demonstrate that the resistance in *S. bulbocastanum* can be captured and passed on to potato breeding lines by the use of somatic hybridization.

MATERIALS AND METHODS

Potato and related species used for somatic hybridization were obtained from Dr. John Bamberg and his

colleagues at the Inter-Regional Potato Introduction Station (NRSP-6), 4312 Highway 42, Sturgeon Bay, WI. These include *S. bulbocastanum*, PI 243510 and *S. tuberosum* PI 23900 (potato). Elite copies of potato cultivars (Katahdin, Atlantic) were obtained from the Wisconsin potato certification program. All cultivars and wild species, and test materials were routinely maintained clonally *in vitro* as described by Haberlach et al (1985). The individual clones were multiplied *in vitro* for analyses.

Protoplasts were isolated from leaves of *in vitro* shoots as described by Haberlach et al. (1985). Somatic hybridizations with the protoplasts were performed using a polyethylene glycol (PEG) protocol. For the most part, the procedure of Austin et al. (1985) was followed. However, after PEG additions, dilutions and pelleting of the cells after the fusion attempts, the cells were suspended in 0.3 M sucrose rather than 0.6 M mannitol. The cell suspension was gently shaken (40 RPM) for 45 minutes and then centrifuged (HNII centrifuge, IEC) at 1300 rpm for 10 minutes in a Babcock bottle. This modification resulted in viable protoplasts and fused cells being concentrated at the surface of the sucrose solution in the bottle, thus separating the viable cells from the pelleted debris.

The resulting fused cells were regenerated into whole plants in a manner similar to that reported by Haberlach et al. (1985). Initially, the cells were plated onto culture medium (CUL, Haberlach et al. 1985) and, after macroscopic calli had appeared, the calli were transferred to differentiation medium (DIF, Haberlach et al. 1985). After 2-3 weeks, the calli were transferred to the differentiation medium developed by Lam (1977). After buds had formed, the calli were transferred to proliferation medium (PM, Haberlach et al. 1985). Shoots that formed were then excised and rooted on standard propagation medium (PROP, Haberlach et al.

1985) and maintained in test tubes *in vitro*. Clonal copies of the reference copy were made for experiments.

DNA extractions and restriction fragment length polymorphism (RFLP) analyses were carried out as described by Williams et al. (1990). Chromosome specific tomato genomic (TG) and cDNA (CD) probes were obtained from Dr. Steve Tanksley, Cornell Univ. Four putative somatic hybrids were analyzed by this method. To complete the analyses for hybridity, randomly amplified polymorphic DNA (RAPD) analyses were carried out as described in greater detail in Example 2. A total of 109 primers (from 380 primers tested) were selected that give clearly scorable polymorphisms between potato and *S. bulbocastanum* . Several of these were used with each of the putative somatic hybrids.

Three of the hybrids have been used extensively in further experiments. These were designated J101, J103, and J138. In crosses, the potato parent was designated with K (for Katahdin) or A (for Atlantic). Thus, for example, J101K1 was the first seed germinated from a berry obtained from the cross of J101 and Katahdin. Similarly, J101K6 and J101K27 were the seedlings obtained from seed 6 and seed 27 respectively from that cross. The BC2 progeny were named by adding the seed number and cultivar lines in that cross. Thus the cross designated as J101K6A22 is the 22nd seedling from the cross of line J101K6 with Atlantic. This shortcut avoided use of the longer and less informational term ((*S. bulbocastanum* + *S. tuberosum*) x Katahdin)) x Atlantic that could be applied to this individual.

Comparisons were made of susceptible and resistant plants in the field. For these studies, the percentage of leaves showing late blight lesions was recorded at various times during the growing season. For detailed greenhouse studies, a modified Horsfall-Barret rating scheme was used to estimate percent leaf infection by *P. infestans*. The ratings and the ranges of %

infections associated with these scores are as follows: 9, no visible infection; 8, <10%; 7, 11-25%; 6, 26-40%; 5, 41-60%; 4, 61-70%; 3, 71-80%; 2, 81-90% 1, >90%; 0, 100% infection.

RESULTS

In total, 80 shoots were obtained from 23 calli. Twenty four of these plants (from 5 calli) showed obvious morphological differences from either of the parent species. The other 56 plants appeared very similar to potato. Initially, chromosome-specific restriction fragment length polymorphism (RFLP) markers were used to confirm that four of the plants derived from fusion of *S. bulbocastanum* and potato cells were indeed somatic hybrids. Prominent potato bands were retained by the hybrids in addition to the diagnostic *S. bulbocastanum* bands (Figure 1). The rest of the potentially hybrid plants were evaluated with RAPD probes (see Example 2). A total of 13 more somatic hybrids were confirmed by these techniques. The confirmed hybrids were derived from four different callus pieces, and thus probably from four different fusion events.

The appearance of leaves and stems of the parent plants and representative somatic hybrids was examined. As has been the case with many of our other hybrids, traits of both of the parent species could be seen in the hybrids. In this case, the purple coloration of the *S. bulbocastanum* stems was expressed in the hybrids. However, leaves of the hybrids were compound rather than single as was the case of the wild species.

Crosses of four of the somatic hybrids were undertaken with the potato cultivars "Katahdin" and "Atlantic" to test for fertility of the hybrids. Each of the tested hybrids yielded viable seeds and sexual progeny. Further crosses of selected individuals from these progeny lines were also successful. Thus, parental lines and two successive backcross populations were

available for evaluation of disease resistance.

Preliminary laboratory tests for resistance to *P. infestans* were made with detached leaves or leaf discs indicated that the somatic hybrids and some of the progeny retained at least some of the resistance to late blight that was shown by the *S. bulbocastanum* parent.

These preliminary laboratory results were confirmed in field tests during a first growing season. Somatic hybrids as well as progeny from somatic hybrids between *S. bulbocastanum* and potato, showed remarkable resistance and were clearly noticeable in test plots as "green islands " in a brown background of dead potato lines. Although the cultivars Atlantic, Russet Burbank, and Snowden were killed, eleven different experimental lines showed less than 10% infection. In each case, the live test plants were surrounded with the susceptible cultivar, Russet Burbank, which had been killed by the fungus by August 9 of that growing season. In contrast, % foliage infections on August 15 were 5.0% and 7.8% for J101K27 and J101K6A22, respectively.

Fourteen BC_1 and BC_2 lines were tested in Toluca, Mexico in a second growing season the following summer. Good resistance in the Toluca field test was also obtained with all lines that were resistant in Wisconsin in the previous summer.

Additional field experiments were carried out at Hancock, Wisconsin, in a third growing season. Again, a severe natural late blight epidemic was obtained in test plots and yields of common cultivars were severely depressed by the late blight epidemic. For example, in plots where an effective fungicide spray regime was maintained, Russet Burbank yields were as high as 1.7 kg/plant. In the yield trial where no fungicide was used, this was cut almost in half to 0.86 kg/plant. One of the *S. bulbocastanum* -derived lines , J103K7, topped all 90 test lines with a yield of 1.36 kg/plant and J138A12 ranked fourth at Hancock in the third growing

season, with a yield of 1.32 kg/plant.

To test the resistance of potentially segregating BC_1 and BC_2 populations, a facility was constructed in the new research greenhouses at the University of Wisconsin Biotron. There, close control of humidity and temperature made uniform epidemics possible. Segregation of resistance and susceptibility was obtained for each of six BC_1 from four different somatic hybrids . Representative tests from one of these lines are shown in Table 1. Three of these lines were further crossed to Atlantic or Norland. Representative results on these BC2 lines are included in Table 2. Again,clear segregation of resistance and recovery of both parental extremes of susceptibility and resistance were obtained with these lines.

Table 1. Representative data from Biotron tests of BC_1 lines for late blight resistance

Plant line	Average Blight score		
	5day	8 day	12 day
J101K09	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
J101K27	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
J101K06	8.8 ± 0.5	9.0 ± 0.0	8.8 ± 0.4
J101K10	9.0 ± 0.4	8.8 ± 0.4	8.6 ± 0.9
J101K30	9.0 ± 0.0	9.0 ± 0.0	8.4 ± 0.9
J101K16	8.8 ± 1.0	8.8 ± 0.5	7.8 ± 1.0
J101K25	8.6 ± 1.1	8.4 ± 0.9	8.2 ± 1.1
J101K02	8.6 ± 0.4	8.0 ± 1.2	7.8 ± 0.4
J101K20	8.6 ± 0.7	7.4 ± 0.9	7.0 ± 0.7
J101K33	7.4 ± 1.8	5.4 ± 0.9	6.2 ± 1.8
J101K19	6.8 ± 2.4	5.6 ± 2.7	5.2 ± 2.4
J101K11	6.0 ± 1.3	4.6 ± 1.1	4.2 ± 1.3
J101K12	7.2 ± 2.5	5.2 ± 2.4	3.8 ± 2.5
J101K18	6.8 ± 1.8	4.6 ± 1.3	3.6 ± 1.8
J101K21	5.2 ± 1.3	2.2 ± 1.3	0.6 ± 1.3
S. bulbocastanum	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
Somatic hybrid J101	8.6 ± 0.5	7.8 ± 0.8	8.2 ± 0.0
S. tuberosum PI 203900	7.0 ± 0.0	5.8 ± 1.3	5.6 ± 1.9
S. tuberosum cv "Kathadin"	4.8 ± 0.4	2.0 ± 0.7	1.0 ± 1.2

Table 2. Exampl s of segregation for late blight resistance in BC_2 progeny of a cross between BC_1 line J101K6 and *S. tuberosum* cv. Atlantic.

Plant line	Average blight score		
	7 day	10day	15 day
J101K6A21	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
J101K6A4	8.8 ± 0.4	8.8 ± 0.4	9.0 ± 0.0
J101K6A22	9.0 ± 0.0	9.0 ± 0.0	8.8 ± 0.4
J101K6A2	9.0 ± 0.0	9.0 ± 0.0	8.8 ± 0.4
J101K6A3	5.2 ± 3.1	5.4 ± 3.6	2.0 ± 3.9
J101K6A10	3.4 ± 2.1	3.0 ± 2.0	1.4 ± 1.9
J101K6A50	5.6 ± 3.4	3.4 ± 3.6	0.0 ± 0.4
J101K6A24	2.6 ± 0.9	1.4 ± 0.5	0.0 ± 0.0
S.bulbocastanum PI 243510	9.0 ± 0.0	8.8 ± 0.4	9.0 ± 0.0
S. tuberosum PI 203900	4.0 ± 1.0	4.0 ± 1.0	0.6 ± 0.9
J101*	7.8 ± 1.1	8.6 ± 0.5	8.4 ± 0.9
Kathadin*	4.4 ± 0.5	4.2 ± 0.8	3.4 ± 1.8
J101K6**	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
Atlantic**	3.6 ± 0.5	3.0 ± 0.0	1.8 ± 1.8

* Lines crossed to generate BC_1 lines
** Lines crossed to generate BC_2 lines.

These results indicate that somatic hybrids between *S. bulbocastanum* and potato are sources of highly effective resistance against late blight. Furthermore, this trait can be passed on to potato breeding lines by conventional sexual crossings. The resistance carries over at least two generations of sexual progeny and, with the third season results just obtained, has been stable for 4 different years in several different locations. As no North American cultivar currently has adequate resistance against this disease, these lines will be very useful for introducing resistance into commercial lines.

The resistance to *P. infestans* from *S. bulbocastanum* appears to be more general than the race-specific resistance derived from *S. demissum* (Black & Gallegly, 1957). Nearly every race of the fungus is known to be found in Toluca, Mexico and was actually isolated from the fields where the progeny of the somatic hybrids showed good resistance. Observations of the

foliage in Toluca in two different growing seasons indicated that some lesions actually formed and that limited sporulation also occurred. Although the disease resistance is highly effective, it is unclear, as yet, the numbers of genes involved. For each of the somatic hybrids tested, the disease resistances of BC_1 lines appear to segregate. Thus, it appears that the late blight resistance gene(s) is (are) heterozygous in the clone of *S. bulbocastanum* utilized in somatic hybridization.

EXAMPLE 2
Identification of RFLPs and a RAPD Marker Linked to Late Blight Resistance Derived from *Solanum bulbocastanum*-Potato Somatic Hybrids

Example 1 described the production of potato-*S. bulbocastanum* somatic hybrids and their progeny, which are resistant to potato late blight. This example describes the identification of DNA markers that co-segregate with disease resistance in these progeny.

Detailed genetic mapping of traits in potato has become possible due to the completion by Bonierbale et al. (1988) of a restriction fragment length polymorphism (RFLP) map in potato. By using tomato clones that had been mapped previously in tomato, a potato map was constructed via a diploid, interspecific cross. The tomato map and the potato map exhibit a nearly collinear gene order (Tanksley et al., 1992).

An alternative method for generating markers is the use of randomly amplified polymorphic DNAs (RAPDs). This technique uses DNA polymerase, synthetic oligonucleotides (10-mers) and genomic DNA mixed together in a PCR thermal cycler to generate bands that are copies of sequences of the genomic DNA present in the mix (J.G.K. Williams et al., 1990). The procedure usually generates between about five and eight specific bands that can be followed, as dominant markers, through various crosses. Moreover, if specific bands can be

linked to a characteristic (such as disease resistance) or to a particular chromosome, or both, the bands can be excised and amplified, then used as a standard RFLP. The method works well to evaluate the extension of DNA introgression from a wild species into a cultivated species. However, RAPDs are species specific, so it is necessary to develop a set for each different species, unlike with RFLPs where inter-species synteny applies.

MATERIALS AND METHODS

Somatic hybrids *S. bulbocastanum* (PI 243510) and *S. tuberosum* (PI 203900) were generated by the method of Austin et al. (1985 and 1993), as describe in detail in Example 1. Plants were derived from fertile hybrids, and three lines of these were designated J101, J103 and J138, respectively. The somatic hybrid plants were crossed as the female parent with *S. tuberosum* cv Katahdin (KAT) to give BC_1 progeny. Three BC_1 progeny were crossed as seed parents with three different potato breeding lines (Norland, Atlantic and A 89804-7) to generate BC_2 populations.

For RFLP and RAPD analysis, DNA was isolated exclusively from plants maintained in axenic *in vitro* cultures. DNA manipulations, PCR amplification protocols for RAPD markers (Williams et al, 1990) and assignment of RAPD markers to *S. bulbocastanum* chromosomes by reference to RFLP markers was performed as described by McGrath et al. (1994), with the exception that a modified thermocycle profile was used, as described by McGrath et al. (1996). RAPD markers were named by the deca-nucleotide primer (obtained from Operon Technologies) and the size of the amplified fragment in subscript (e.g., a 586 bp fragment amplified by primer GO2 is represented as $GO2_{586}$).

Segregation of RAPD markers was also analyzed with maximum likelihood algorithms contained in the MapMaker computer package (Lander et al., 1987).

MapMaker version 2.0 for the MacIntosh was used. The data were coded and analyzed as a "Haploid" population under the "Data Type" option. The use of MapMaker for the analysis of interspecific somatic hybrids is non-standard and does not provide three-point linkage data. However, recombination frequencies do have meaning in this context. Markers that show identical segregation have a recombination frequency of 0.0%. Markers that deviate from complete co-inheritance by a single change (e.g. a marker is present in one additional individual) show a recombination frequency proportional to the population size; in this instance 1/101 individuals or 1.0%. Thus, multiples of this value indicate the number of differences observed between any pair of markers.

RESULTS

Using RAPD marker analysis, twelve synteny groups were established for *S. bulbocastanum*, corresponding to the base number of chromosomes in that species. The groups were associated with chromosomes by comparative RFLP analysis. A MapMaker analysis of RFLPs and RAPD fragments of synteny group A (chromosome 8) of *S. bulbocastanum* is shown in Figure 2. This synteny group contains the RAPD markers $GO2_{586}$ and $PO9_{587}$ as well as the RFLP CT88. It appears that the resistance area (R) is flanked by CT88 and $GO2_{586}$ on one side and $PO9_{587}$ on the other (Figure 2).

The nucleotide sequence of $GO2_{586}$ is set forth herein as SEQ ID NO: 1. The nucleotide sequence of $PO9_{587}$ is set forth herein as SEQ ID NO:2. Three nucleotide sequences of CT88 are set forth herein. SEQ ID NO:3 is the sequence published by Tanksley et al. (http://probe.nalusda.gov.8300/cgi-bin/browse/solgenes); SEQ ID NO:4 is from R4 potato, and SEQ ID NO:5 is from *S. bulbocastanum* (PT29). Slight differences were noted among the three sequences. The R4 potato marker is 589 bp in length, while the *S. bulbocastanum* RFLP is 592 bp

SUB B3

SUB B3 cont

and the Tanksley et al. sequence is 596 bp. In addition, the *S. bulbocastanum* CT88 homolog possesses two TaqI sites, whereas the other two have only one.

The fusion of *S. tuberosum* with *S. bulbocastanum* yielded 17 confirmed somatic hybrids. The somatic hybrids are quite resistant to early blight and to late blight. The progeny of some crosses were found to segregate for high resistance to both early blight and late blight. Some of the progeny are highly resistant even to fungal lines that are highly virulent, complex races. Several highly resistant clones were selected and further crossed with potato. Three sets of BC_2 mapping populations have been generated by crosses with potato cultivars. Field studies in Wisconsin first indicated resistance to *Verticillium*, early blight and late blight in these materials. Subsequent studies on late blight resistance were carried out in North Dakota, Prince Edward Island, Washington state, New York, Maine, West Virginia and Toluca, Mexico. The Mexico and Wisconsin studies were repeated for a second and third season and late blight resistance in these materials appears to be durable.

Segregation of late blight resistance in selected BC_1 and BC2 populations were shown in Example 1, Tables 1 and 2. Table 3 below shows results of segregation analysis of BC_2 progeny with respect to late blight resistance in relation to the presence or absence of RAPD marker $GO2_{586}$ for Chromosome 8 of *S. bulbocastanum*. The data shown in Table 3 were generated by PCR amplification, followed by agarose gel electrophoresis and ethidium bromide staining to observe the presence or absence of an amplified 586 bp band.

Table 3. Segregation of BC2 progeny in relation to the presence or absence of RAPD marker $GO2_{586}$.

Clone	Late blight rating			Marker
	7 day	10 day	15 day	
PI 245310 (BLB)	9.0	8.8	9.0	+
PI 203900 (TBR)	4.0	4.0	0.6	-
J101	7.8	8.6	8.4	+
Kathadin	4.4	4.2	3.4	-
J101K6	9.0	9.0	9.0	+
Atlantic	3.6	3.0	1.8	-
J101K6A22	9.0	9.0	8.8	+
J101K6A50	5.6	3.4	0.0	-
J101K6A32	8.6	8.4	9.0	+
J101K6A38	2.8	3.4	1.4	-
J101K6A21	9.0	9.0	9.0	+
J101K6A07	6.2	7.0	5.3	-
J101K6A12	9.0	9.0	9.0	+
J101K6A03	5.2	5.4	2.0	-
J101K6A19	9.0	9.0	8.8	+
J101K6A18	7.0	7.7	5.3	-
J101K6A02	9.0	9.0	8.8	+
J101K6A54	5.0	3.2	1.0	-

As can be seen from Table 3 (as well as other results not shown), late blight resistance in the BC_2 clones is highly correlated (>95%) with the presence of RAPD marker $GO2_{586}$, which has been keyed to chromosome 8 of *S. bulbocastanum*. This high correlation of the resistance phenotype with the marker indicates that the gene(s) responsible for conferring late blight resistance reside on the chromosome at or near the $GO2_{586}$ marker. Accordingly, the marker can be used as a molecular tag to follow resistance through breeding programs and to identify and isolate disease-resistant breeding stock. Additionally, knowing the relative proximity of the

resistance gene(s) to the marker, isolation of the gene(s) will be facilitated.

The RFLP and RAPD markers CT88 and $PO9_{587}$ discussed above can also be used as molecular tags to follow resistance through breeding programs. The use of a combination of the resistance-associated RAPD and RFLP markers can provide an added advantage in following segregation in breeding, and eventually in isolating and cloning the *S. bulbocastanum* gene(s) responsible for conferring resistance to late blight and other diseases.

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The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.